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Optimizing Transposon Mutagenesis in Vibrio Strains to Identify Genes Involved in Antibiotic Production

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Abstract

Based on previous studies that have shown the competitive nature of non-pathogenic environmental strains of *Vibrio*, we hypothesize that environmentally derived bacteria can inhibit *Vibrio* pathogens, and possibly be a source of novel antibiotics. A previous experiment performed in the lab tested environmental *Vibrio* strains from various habitats against the pathogenic strains, *Vibrio cholerae* and *Vibrio parahaemolyticus*. Of the 3,456 strains collected, members of the Wildschutte lab identified 102 environmental strains of *Vibrio* that inhibited the growth of both pathogens. The data suggests that environmental *Vibrio* strains directly inhibit the growth of related pathogens. Our project involves the identification of genes responsible for producing secondary metabolites in order to discover potential novel antibiotics. To accomplish this task, all 102 strains will be screened for their ability to undergo conjugation and transposon mutagenesis. Candidates efficient in these processes will be subjected to a large scale mutant hunt to identify genes involved in antibiotic production.

Introduction

The emergence of pathogens resistant to all known antibiotics has become an increasingly prevalent and dangerous problem. Presently, it is estimated that about 700,000 people worldwide are killed annually as a result of antimicrobial resistant infections (O'Neil 2014). By 2050, deaths from antibiotic resistance bacteria in the United States alone is predicted to be 300,000 per year and the leading cause of death worldwide (Frieden 2013). Thus, the evolution of antibiotic resistant pathogens is a major concern. Making the crisis worse, is that pharmaceutical companies have stopped drug development due to low profitability, thereby leaving antibiotic discovery to university research laboratories.

The Wildschutte laboratory uses a unique bacterial population-level approach to assist in drug discovery to help combat this imminent global threat of resistance. Populations are groups of closely related strains (e.g. a species). Environmental strains of bacteria are used as sources for novel antibiotics. For this study, aquatic strains of *Vibrio* are used primarily due to their genetic diversity and ability to inhibit the growth of pathogenic *Vibrio*, meaning environmental *Vibrio* produce compounds that inhibit the growth of pathogens (Cordero *et al*, 2012). *Vibrio* is found on and within many aquatic organisms, such as crabs, mussels, and zooplankton, and on suspended nutrient particles in the ocean, all which influence population diversity due to the ability to grow in association with different hosts or habitats (Preheim *et al*, 2011 and Burks *et al*, 2017).

Previous research in the Wildschutte laboratory has shown the ability of *Vibrio* strains to inhibit dangerous pathogens, such as *Vibrio cholerae* and *Vibrio parahaemolyticus*. Both species cause severe gastrointestinal diseases. *V. cholerae* causes cholera in which patients may produce 3 to 5 US gallons of diarrhea a day. There are 1.3 million to 4.0 million cases of cholera per year,

and 21,000 to 143,000 deaths worldwide due to cholera. Patients are infected by the fecal-oral route from contaminated food or water caused by poor sanitation. Most cases occur in under-developed countries where the water is not sanitized. Severe cases need treatment for dehydration and a course of antibiotic therapy (Ali *et al*, 2015). Infections by *V. parahaemolyticus* are usually less severe than those caused by *V. cholera*e. Typically, antibiotics such as doxycycline or chloramphenicol are used to combat these infections; however, many *Vibrio* pathogens have become resistant to these combative efforts (Burks *et al*, 2017). Therefore, it is imperative to discover novel sources of antibiotics in order to prevent these devastating illnesses.

To test if environmental *Vibrio* strains can inhibit *V. cholerae* and *V. parahaemolyticus*, the Wildschutte lab sampled water off the coast of Nahant, Massachusetts and isolated 3,456 environmental *Vibrio* strains from 63 µm, 5 µm, and 1 µm size fractionated particles and free-living in the water column and over a three month time span. A high-throughput antagonistic plate assay was performed, and it competed all 3,456 strains against a panel of five *Vibrio cholerae* and three *Vibrio parahaemolyticus* pathogenic strains, resulting in a total of 27,648 individual interactions. From all the interactions, 102 environmental strains inhibited the growth of one or more of the *Vibrio* pathogens.

Based on preliminary data collected and prior research, we hypothesized that aquatic *Vibrio* strains possess genes involved in antibiotic production. To test our hypothesis, we planned to: (1) optimize conjugation efficiency of the 102 strains that displayed antagonistic ability, (2) identify an environmental *Vibrio* strain sensitive to each of the *Vibrio* strains efficient in conjugation, and (3) perform a large scale mutant hunt with the *Vibrio* strains that were efficient in conjugation and transposon mutagenesis.

Preliminary data

The three main aspects of the Wildschutte laboratory's method for discovering novel antibiotics are (i) strain isolation, (ii) antibiotic production, and (iii) genetic characterization. Previous research has genetically characterized and identified 102 strains able to inhibit the growth of *Vibrio* pathogens. This information enabled us to perform conjugation and transposon mutagenesis on the 102 antagonistic strains in order to identify genes involved in antibiotic production.

(i) Strain Isolation. *Vibrio* are marine-derived bacteria, so they can easily be isolated from coastal and aquatic habitats. Coastal water was passed through 63 µm, 5 µm, and 1 µm size filters to capture bacteria associated with different sized particles and free-living in the water column over a three-month period: August 10th, September 18th, and October 13th of 2010.

Table 1. Environmental strains used in the study and their activity.

Habitat	No. of strains isolated (antagonistic activity)			Total strains
	Aug 10th	Sept 18th	Oct 13th	
63 µm particle	288 (3)	288 (11)	288 (6)	864 (20)
5 µm particle	288 (6)	288 (3)	288 (4)	864 (13)
1 µm particle	288 (3)	288 (12)	288 (5)	864 (20)
Free-living	288 (5)	288 (30)	288 (14)	864 (49)
Total strains	1,152 (17)	1,152 (56)	1,152 (29)	3,456 (102)

Table 1. Distribution of strains based on the filtration (habitat) and month isolated.

The filters are taken and placed on Thiosulfate-Citrate-Bile Salts-sucrose agar plates. The plates were incubated at room temperature for 24 hours, or until single, isolated colonies appeared. The strains were streaked out on Tryptic Soy Broth with 20% NaCl (TSB2) agar media to obtain a pure and single strain of *Vibrio*. The strains were stored in 96-well stocks, which are frozen at minus 80° Celsius, so that they may be used in the future.

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(ii) Antibiotic production. The strains were tested for their inhibitory activity using an antagonistic assay. For an antagonistic assay, 20 µl of one of eight *Vibrio* pathogens (Table 2) was spread plated onto a TSB2 plate, and one µl each of 96 environmental *Vibrio* strains was stamped onto the plate. The environmental and pathogenic bacteria were co-grown for 24 hours at 23° C and subsequently scored for antagonistic activity. This method creates interactions between each of the environmental strains and the pathogen, allowing for analysis of inhibition.

Table 2. Pathogenic strains used in the study.

Pathogen	Description
V. cholerae N16961	Serotype O1 El Tor, Bangladesh in 1971
V. cholerae MO10	Serotype O139 El Tor; India in 1992
V. cholerae O395	Serotype O1 Classical; India in 1965
V. cholerae VO-146	Serotype O10; Bangkok in 1993
V. cholerae VO-258	Serotype O8; Bangkok in 1993
V. parahaemolyticus EB101	Shirasu food-poisoning; Japan in 1965
V. parahaemolyticus BB22OP	Strain LM5312; Bangladesh in 1980s
V. parahaemolyticus 954	Clinical isolate

Table 2. List of *Vibrio cholerae* and *Vibrio Parahaemolyticus* strains used.

An indication of inhibitory activity is a zone of inhibition, an area of no bacterial growth, surrounding the circular region of the environmental strain (Figure 1). Each of the 3,456 environmental strains isolated (Table 1) was tested for their antagonistic ability against each of

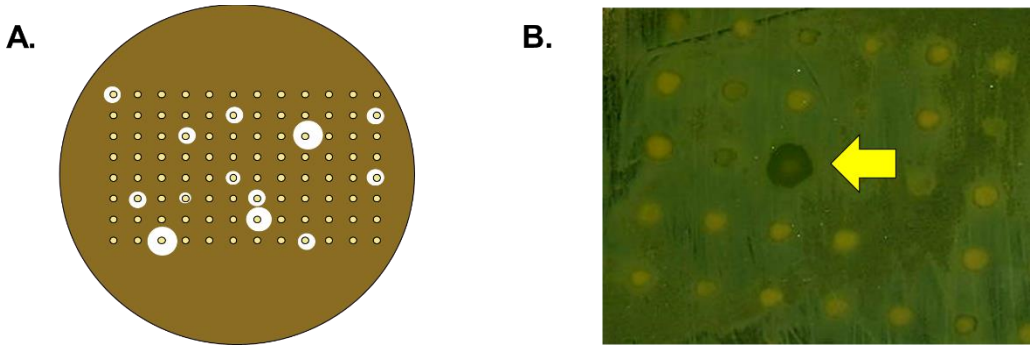

Figure 1. The antagonistic assay. (A) Cartoon showing antagonistic activity and different sizes of the zones of inhibition (B) Photograph showing antagonistic activity of environmental isolates against pathogenic *Vibrio* (yellow arrow).

the eight pathogens (Table 2), creating a total of 27,648 individual interactions. 102 strains displayed inhibitory activity (Table 1).

(iii) Genetic Characterization of Antagonistic Strains. The antagonistic strains (Table 1) were genetically characterized using polymerase chain reaction (PCR) in order to create a phylogenetic tree showing the relatedness among every strain. The gene used to characterize the strains was *hsp60*, whose product involves protein folding within *Vibrio* (Burks *et al*, 2017). *Hsp60* is a housekeeping gene and has regions of high variability between the highly conserved

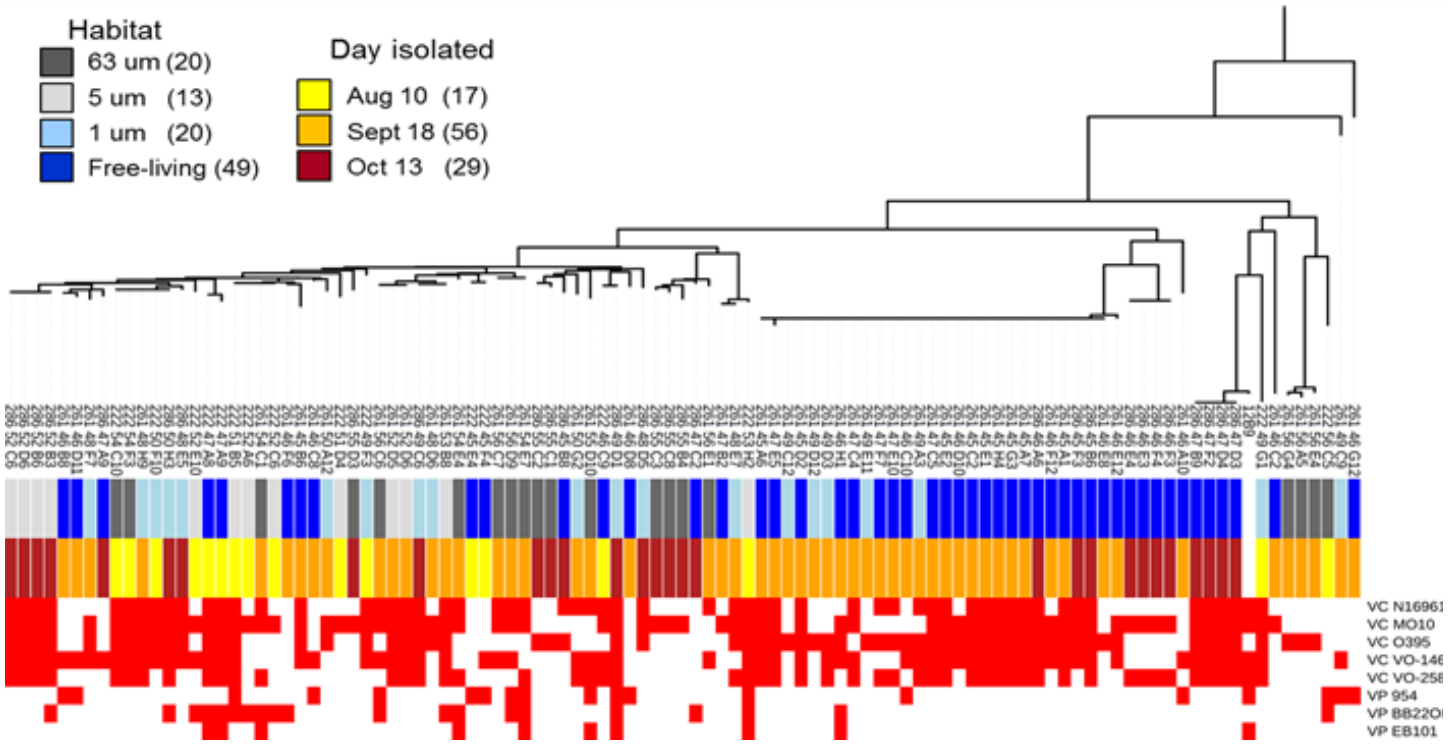

Figure 2. Phylogenetic tree. The antagonistic data is shown on the bottom; the 8 *Vibrio* pathogens are listed next to the inhibitory activity data (VC refers to *Vibrio cholerae* and VP refers to *Vibrio parahaemolyticus*). The day the strains were isolated is identified above the antagonistic activity data by the yellow (August), orange (September), and maroon (October) bars. The habitat from where the environmental *Vibrio* are isolated from is shown by the dark gray (63 µm), light gray (5 µm), light-blue (1 µm), and blue (free-living) bars. All of the strains are compiled at the top of the tree with clades showing relatedness among them.

regions of the gene, which allows us to differentiate and compare the species being sequenced.

The *hsp60* gene of all strains was amplified, sequenced, and aligned. The alignment was used to

create a Neighbor-Joining phylogenetic tree. The data from the antagonistic assays was compiled onto the phylogenetic tree (Figure 2).

Material and Methods

Each of the 102 strains that displayed inhibitory activity was tested for conjugation and transposon mutagenesis efficiency, which consisted of the majority of my work with the *Vibrio* strains. Conjugation is the process by which a plasmid is transferred from one bacterium to another bacterium. The bacterial strain EC114, an *E. coli* strain, contains the pSCAT vector, which carries the transposon with the chloramphenicol resistance gene (Cordero *et al*, 2012). The pSCAT vector from EC114 is conjugated to the recipient *Vibrio* strain, followed by transposition. This process is integral in the discovery of genes involved in antibiotic production because the transposon will insert itself in any region of the *Vibrio* genome. The goal is for the transposon to insert itself within the genes involved in antibiotic production.

Media Needed for Experiments. Before conjugation was performed, the necessary materials were made and acquired. Agar plates and liquid media were all made prior to performing conjugation. Tryptic Soy Broth (TSB2) agar plates were used to grow the *Vibrio* strains. The media was made in a two liter Erlenmeyer flask; typically, with one liter of water, 30 grams of tryptic soy broth, 16 grams of agar, and 15 grams of NaCl. The amounts were changed depending on how much media was needed. The flasks were autoclaved for 45 minutes and cooled before pouring into petri plates (Figure 3). TSB2 liquid media was used for making overnight cultures of *Vibrio*. Liquid media contains everything that TSB2 agar plates contain, except agar, which is the substance that solidifies media.

Figure 3. TSB2 media in flasks before pouring.

Antibiotics mixed with different liquid media were also made for different aspects of conjugation. Chloramphenicol, the antibiotic used, came in a stock solution of 34 mg/mL, so it was necessary to calculate the correct amount of chloramphenicol to add to the media to attain the desired concentration. Lysogeny broth (LB) media with 25 µg/mL of chloramphenicol (LB cam25) was used to culture EC114 in both liquid cultures and on agar plates. Chloramphenicol was used to select for the pSCAT vector that carries the chloramphenicol resistance gene in EC114. Another requirement for conjugation was a concentration of 250 µM of diaminopimelic acid (DAP). DAP is a component of peptidoglycan in bacterial cells; EC114 is a DAP auxotroph so this amino acid was added to media for the growth of EC114 (Cordero *et al,* 2012). TSB2 with 12 µg/mL of chloramphenicol was needed to select for transconjugates.

Calculations

Sample calculation for making LB cam25 plates with 1 L of H_2O:

$$M_1V_1 = M_2V_2$$

$$\left(34\frac{mg}{mL}\right)(V_1) = (1000mL)\left(0.025\frac{mg}{mL}\right);\ V_1 = 0.735\ mL\ of\ chloramphenicol$$

Sample calculation for making TSB2 cam12.5 plates with 1 L of H_2O:

$$M_1V_1 = M_2V_2$$

$$\left(34\frac{mg}{mL}\right)(V_1) = (1000mL)\left(0.0125\frac{mg}{mL}\right);\ V_1 = 0.367\ mL\ of\ chloramphenicol$$

Sample calculation for amount of DAP needed for 1 L of H_2O:

$$50\ mM\ *\left(\frac{1\ mol\ DAP}{1000mM}\right)*\left(\frac{9.51\ g\ DAP}{1\ mol\ DAP}\right) = \frac{9.51\ g\ DAP}{1\ L\ H_2O}$$

Growth Conditions. Conjugation was a week-long process that began with streaking out frozen environmental strains of *Vibrio* from the freezer stock of the 3,456 strains of environmental *Vibrio* that were isolated. About five – six strains were tested for conjugation efficiency every week. The strains were stored in tubes containing 800 µL of the TSB2 media containing the environmental strain and 200 µL of 70% glycerol. The tubes had to be placed on dry ice when streaking to prevent the frozen bacteria from melting, which can kill the bacteria. Before streaking, a Bunsen burner was lit to prevent any contamination that might occur, which is in accordance with standard aseptic technique used in all microbiology laboratories. A long toothpick was used to scrape a small amount of the bacteria, which was used to create the

primary streak. A new toothpick was then used to create a secondary and tertiary streak. This ensured the production of isolated colonies (Figure 4). The environmental *Vibrio* strains were streaked onto TSB2 plates. For EC114, 150 µL of 250 mM DAP was spread plated onto an LB cam25 plate. The TSB2 plates were incubated at 23° C for 24 hours; the LB cam25 and DAP plate was incubated at 37° C for 24 hours.

The isolated colonies from the streaked out plates were used to make overnight cultures of the *Vibrio* strains. Overnights were made using test tubes, liquid media and a single, isolated colony from the streaked out strains. For the environmental *Vibrio* strains, the test tubes were filled with five µL of TSB2 liquid media. A large toothpick was used to pick up a small amount of bacteria from an isolated colony, and then inserted into the test tube. The same process was

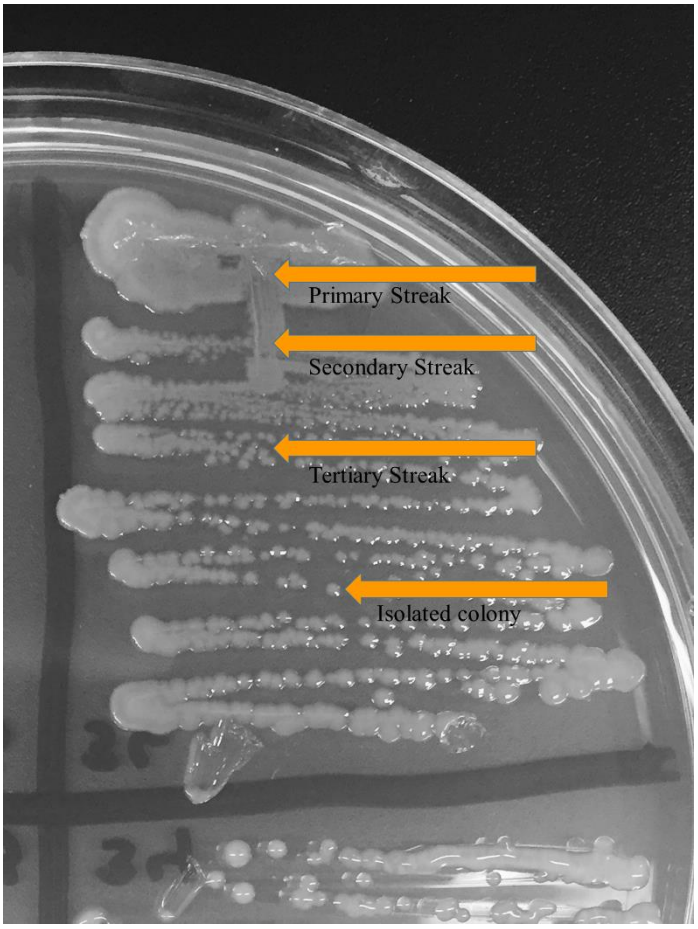

Figure 4. Agar plate with streaked bacteria.

used for EC114, but LB cam 25 and DAP were used as the media, instead of TSB2. Once all the tubes were inoculated, they were placed on a shaker which continuously moved the bacteria so growth was not concentrated in one area. Inoculated cultures of *Vibrio* grew overnight at room temperature; EC114 grew at 37° C.

Conjugation and Transposon Mutagenesis (CTM) Efficiency. Conjugation day one was performed once the overnight cultures grew. One mL of the EC114 culture was pipetted into an Eppendorf tube and centrifuged for five minutes at 12 rpm. This resulted in the formation of a pellet of EC114 that carries the pSCAT vector. The supernatant was removed from the tube, and the tube was suspended with one mL of LB and vortexed to mix the pellet with the media. In a separate Eppendorf tube, 100 µL of one of the environmental *Vibrio* cultures (the recipient strain) was combined with 100 µL of the EC114 culture (the donor strain). Once all of the samples were prepared, they were centrifuged for five minutes at 12 rpm. Once again, a pellet was formed containing the donor and recipient strains. The supernatant was removed and the cells were suspended in 10 µL of liquid TSB2. These cells were spotted onto the surface of a TSB2 plate with 150 µL of 250 mM DAP plate. When testing CTM ability for the first time, a test for natural resistance to chloramphenicol was also done because if the *Vibrio* strain is naturally resistant then it cannot be used for CTM. There would be no way to tell if the bacteria growing on the TSB2 with cam12.5 plate was due to the transposon insertion since it was naturally resistant to the antibiotic. Testing for natural resistance is done the same way as conjugation, but the donor strain was omitted from the addition to the Eppendorf tube. All plates were incubated for 24 hours at 23° C.

Conjugation day two was performed by using the conjugation spots that were made the previous day. The entire growth spot was scraped up using a sterile bent yellow pipette tip. The

spot was transferred to a tube filled with 200 µL of liquid TSB2. 100 µL of the contents were spread plated onto the surface of a TSB2 cam12.5 plate. This was done for every spot as each spot was a different *Vibrio* strain. The plates were incubated at 23° C for about 48 hours.

Identification of a sensitive non-pathogenic strain. Once each of the 102 strains were tested for their ability to conjugate, antagonistic assays were performed. A sensitive strain was needed in order to perform CTM and an upscale mutant hunt. To observe the successful insertion of the transposon within the genes involved in antibiotic production of the *Vibrio* strains, a different environmental strain that is susceptible to the antibiotics being produced by the *Vibrio* strains must be identified. This insertion is observed by the loss of killing phenotype – the lack of a zone of inhibition. After identifying strains efficient in conjugation, the strains were tested against other environmental *Vibrio* strains from the same collection of the 3,456 strains (Table 1) previously used for antagonistic assays against *Vibrio* pathogens. If a sensitive strain is found to any of the five *Vibrio* strains efficient in CTM, a large-scale mutant hunt can be performed. This has to be done on a large scale because *Vibrio* have approximately 5,000 genes in their genome, and we cannot predict where the transposon will insert.

Like conjugation, antagonistic assays took a full week to complete. During the first day, environmental *Vibrio* strains were streaked onto TSB2 plates. The environmental *Vibrio* strains were tested from 96 well frozen stocks (Figure 5). Every week, 12 of the strains were streaked out as there were 12 strains in each row of the block. The *Vibrio* strains efficient in conjugation were also streaked; there were five of them. Plates were incubated at room temperature for 24 hours. The next day, overnight cultures were made by the same process detailed above.

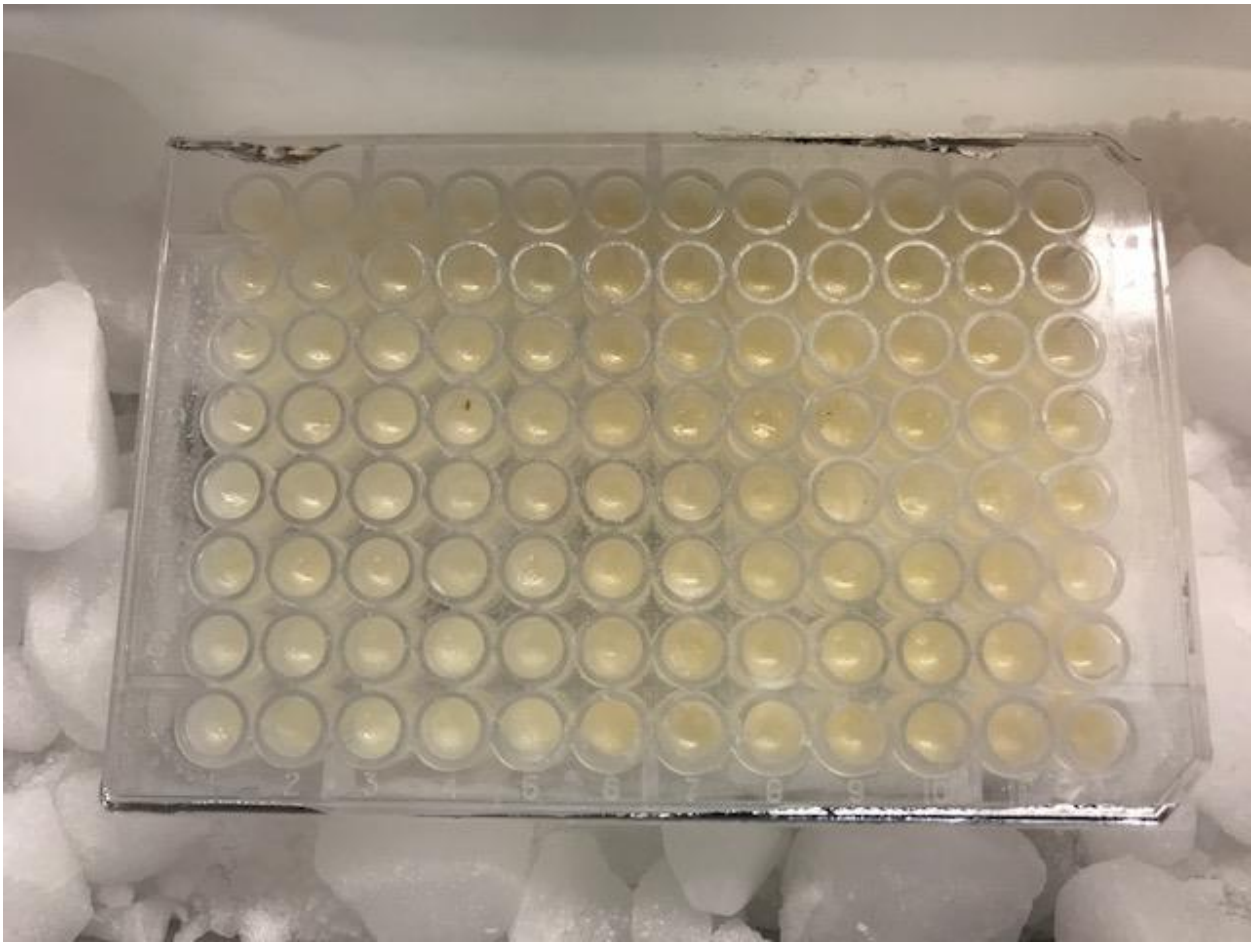

Figure 5. 96 well frozen stock used to store environmental *Vibrio.*

The antagonistic assays were performed after cultures grew for 24 hours. For the assay, 15 µL of one of the 12 environmental *Vibrio* strains was spread plated onto the surface of a TSB2 plate. The plate was split up into five sections in order to test each of the five *Vibrio* strains efficient in CTM. When each of the 12 strains was spread plated, the plates were left for about half an hour so that the plates absorbed the strain. After the environmental strain was absorbed, each of the five *Vibrio* strains was spotted onto the plates. This was done by taking two µL from one of the overnight cultures using a micropipette and spotting it on the corresponding section on the plate. This was performed five times for each of the *Vibrio* strains. Once all of the strains were spotted, the plates were incubated at 23° C overnight and checked for antagonistic activity the following day.

Results

Testing for CTM Efficiency. In order to perform a large scale mutant hunt, the antagonistic strain must exhibit CTM efficiency. *Vibrio* have about 5,000 genes and possible locations where the transposon can insert. For the transposon to insert within genes involved in producing antibiotics, we must create many interactions between the genes of the *Vibrio* and the transposon. It is easiest to optimize growth of the *Vibrio* when it is exhibits CTM efficiency. After testing all of the 102 antagonistic strains (Figure 2), 29 were observed for their ability to undergo CTM. A full lawn of mutants was observed if the strain was efficient in CTM (Figure 6). After verification, five antagonistic strains were identified: 10N.286.46.E3, 10N.286.46.E4, 10N.286.46.F3, 10N.286.47.C2, 10N.286.55.D3, labeled 2, 3, 4, 12, and 19, respectively, when performing tests. Next, a sensitive strain must be identified for the transposon mutagenesis screen. The five antagonistic CTM efficient strains were tested against environmental *Vibrio* strains for susceptibility.

Identify Environmental Strains for use in CTM. Environmental *Vibrio* strains from blocks 10N.286.52, 10N.286.51, and 10N.286.49 were tested in the antagonistic assays in order to identify a sensitive strain that can be used for a large scale mutant hunt. The strains were all isolated on October 13 2010. Strains from blocks 10N.286.52 and 10N.286.51 were isolated from 5 µM filters, while strains from block 10N.286.49 were isolated from 1 µM filters. All of block 10N.286.52 was tested (96 strains), and strains from rows A – C were tested from blocks

10N.286.51 and 10N.286.49 (72 strains). The five antagonistic *Vibrio* strains were tested against a total of 168 environmental *Vibrio* isolates, creating a total of 840 individual reactions.

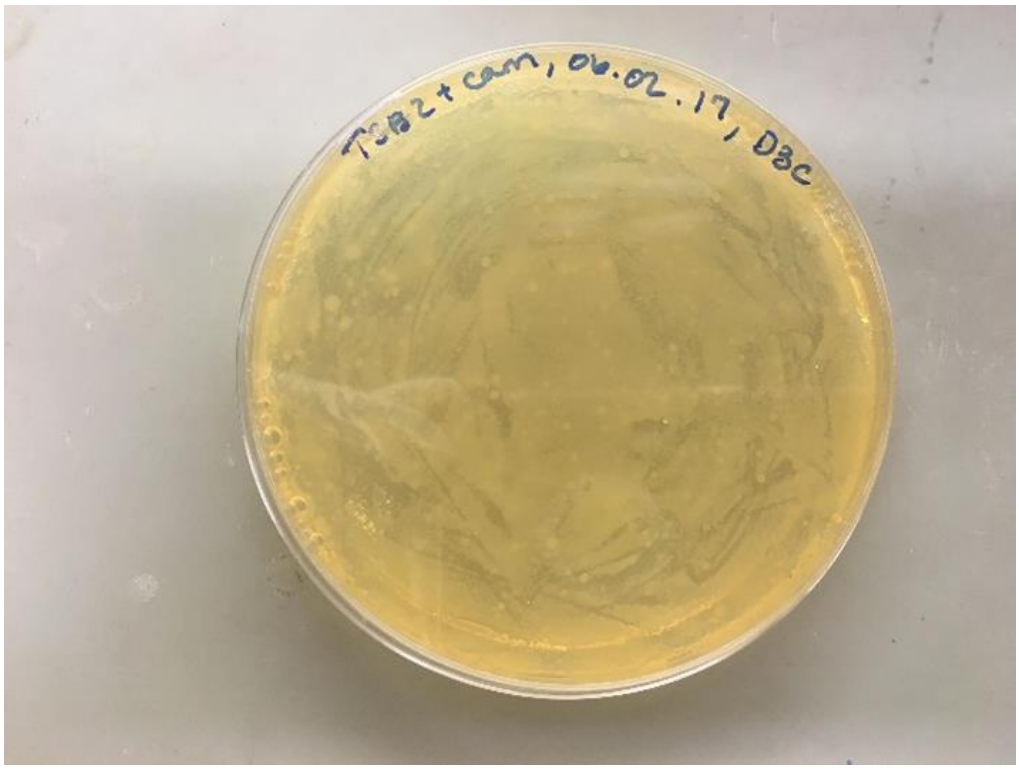

Figure 6. Plate with CTM efficient strain.

After testing each of the five antagonistic, CTM-efficient *Vibrio* strains against the 168 environmental strains for antagonistic activity, we found no environmental strains that were sensitive to any of the five strains. Throughout the process, there were strains that displayed antagonistic activity after doing the spot assay, but with further verification and analysis, no zones of inhibition could be identified with certainty. For example, strain 12 was able to inhibit the growth of F-2 from block 10N.286.52, and a clear zone of inhibition was observed (Figure 7); however, after verifying the strain by performing multiple subsequent antagonistic assays, no zone of inhibition was observed. Furthermore, no reliable results were observed as growth of the *Vibrio* strains was difficult to optimize with regards to the antagonistic assay.

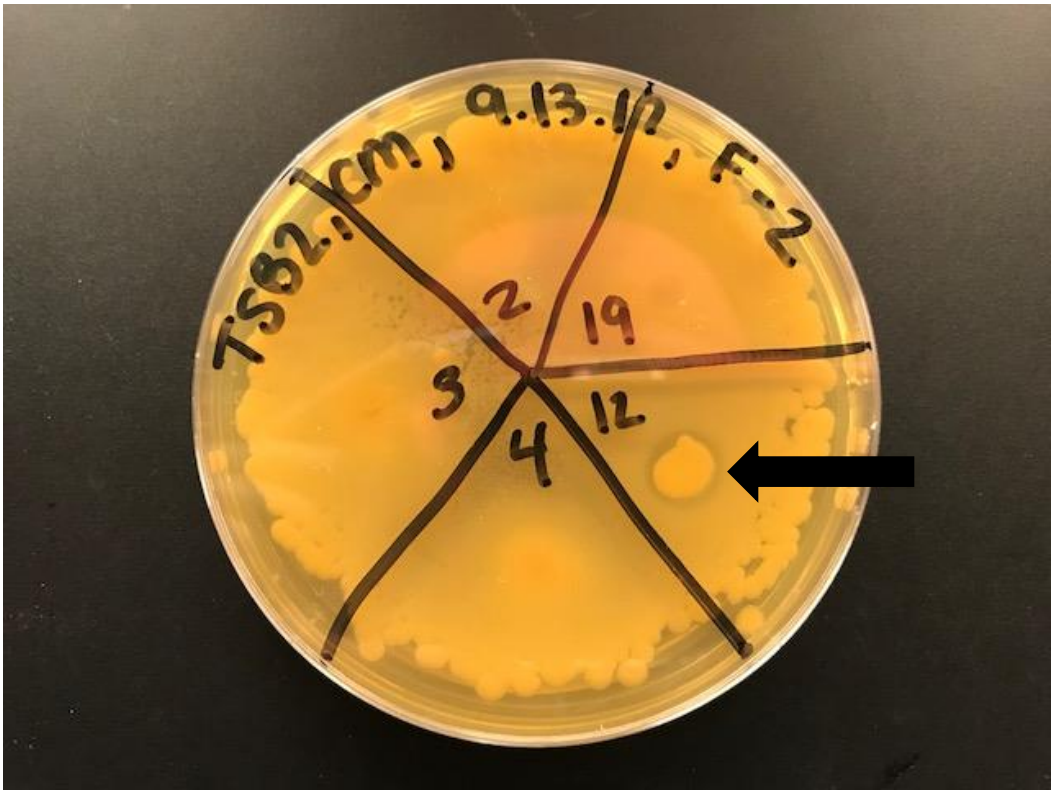

Figure 7. Vibrio strain 10N.286.47.C2 (12) has inhibited the growth of environmental strain F-2.

Discussion

Out of the three goals for this project: (1) test and optimize conjugation efficiency of the 102 strains that displayed antagonistic ability, (2) test and discover an environmental *Vibrio* strain sensitive to each of the *Vibrio* strains efficient in conjugation, and (3) perform a large scale mutant hunt with the *Vibrio* strains efficient in conjugation, we only were able to obtain results from 1 and 2. Although we identified five, of 102 (Figure 2), antagonistic strains that were able to undergo CTM efficiently, no susceptible environmental strains were identified from the 168 strains we tested. Therefore, we could not perform an upscale mutant hunt. A sensitive strain is necessary for CTM because a loss-of-killing mutant must be identified in order to find genes involved in antibiotic production.

A setback that we observed while doing CTM and antagonistic assays was variable results, meaning it was difficult to optimize growth of the *Vibrio* strains with dissimilar results. This may be due to many different factors, including temperature change in the lab. Since the *Vibrio* grow at 23° C, which should be the standard laboratory temperature, they grow on lab bench tops, instead of in an incubator, where they may be exposed to varying temperature. This may cause a difference in how the *Vibrio* strains grow and react to the environment. Another reason why we were not seeing desired results was because we simply did not test enough strains for the antagonistic assays. As the process was very time consuming, taking a full week to complete with 12 strains tested each week, we were not able to test every one of the 3,456 strains. If we had continued further, we may have found a good sensitive strain but lack of time prevented this.

Though we were not able to perform a large scale mutant hunt, we gained some insights that would help further this project in the future. When spread plating for the antagonistic assays, we tried plating different amounts of the sensitive strain: 40 µL, 20 µL, 15 µL, and 10µL. We found that spread plating 15 µL produced the best results; if there was too much of the sensitive strain spread plated then it would grow too fast, and we would not be able to see the spots of the *Vibrio* strain. Conversely, if there was too little of the sensitive strain then we were not able to see proper growth and results.

Since we were unable to find a good sensitive strain, it would be useful to use the susceptible pathogenic *Vibrio cholerae* and *Vibrio parahaemolyticus* strains (Figure 2) in the future. It would be pragmatic to verify that the environmental *Vibrio* strains are able to produce good zones of inhibition, and then do transposon mutagenesis using one of the pathogenic *Vibrio*

as the sensitive strain. We were reluctant to work with the pathogenic *Vibrio* strains in order to avoid lab contamination and potential illness.

Conclusion

While we were unsuccessful in optimizing transposon mutagenesis with strains efficient in CTM, it is still imperative that the search for novel antibiotics continues. An estimated 23,000 people die each year as a direct result of multidrug resistant pathogens in the United States (Frieden 2013). Moreover, this method of identifying genes involved in antibiotic production can be used with other bacteria. Although transposon mutagenesis was unsuccessful with *Vibrio* in our project, it has been successful with a different environmental bacteria, *Pseudomonas*, in the Wildschutte laboratory (Chatterjee *et al*, 2017). Overall, this project can be continued further, but it may be more worthwhile to use *Pseudomonas* as the model organism.

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